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(54) Gene encoding endo-beta-n-acetyl glucosaminidase A

(57) Described are nucleic acid molecules having a sequence encoding a polypeptide possessing endo- β -N-acetylglucosaminidase A activity or functionally equivalent variants thereof and a method for producing a polypeptide possessing endo- β -N-acetylglucosaminidase A activity or functionally equivalent variants thereof by recombinant DNA technology using the described nucleic acid molecules.

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Description

The present invention relates to nucleic acid molecules encoding a polypeptide possessing endo- β -N-acetylglucosaminidase A activity, and to a method for producing a polypeptide possessing endo- β -N-acetylglucosaminidase A activity by the use of the DNA.

In recent years, the various physiological functions of the sugar chain moieties of molecules known as complex carbohydrates, such as glycoproteins and glycolipids, have drawn attention. At present, carbohydrate-decomposing enzymes serve as very useful tools for elucidation of the structure and biological activity of sugar chains. Endo- β -N-acetylglucosaminidase, in particular, catalyzes the reaction in which the GlcNAc β 1-4GlcNAc bond of di-N-acetylchitobiose at the reduction end of the N-linked sugar chain of glycoproteins is broken to cut off the sugar chain from the protein and leave N-acetylglucosamine on the protein side, and has been used for structural or functional analysis of glycoproteins.

Also, some forms of endo- β -N-acetylglucosaminidase are known to catalyze sugar chain rearrangement reactions; endo- β -N-acetylglucosaminidase A from the *Arthrobacter protoformiae* AKU 0647 strain (hereinafter also referred to as Endo-A), in particular, has been reported to possess very potent sugar chain rearrangement activity (Japanese Patent Laid-Open No. 5-64594). Specifically, Endo-A efficiently catalyzes the reaction in which the N-binding oligomannose type sugar chain of glycoproteins is cut out and transferred to an acceptor carbohydrate or complex carbohydrate. The Endo-A enzyme is therefore very useful not only for the structural analysis of sugar chains of glycoproteins but also for other purposes such as modification of sugar chains of complex carbohydrates, and preparation of neoglycoproteins.

A known form of Endo-A is derived from *Arthrobacter protoformiae* [Applied and Environmental Microbiology, 55, 3107-3112 (1989)].

However, in the method in which *Arthrobacter protoformiae* is cultured to obtain Endo-A, proteases and other glycosidases are also produced. It has been difficult to separate and purify these co-present enzymes from Endo-A. Also, to induce Endo-A enzyme production, ovalbumin or a sugar peptide thereof must be added to the culture medium. There has therefore been a need for the development of a method enabling the production of highly pure Endo-A at low cost.

Although purification of Endo-A from *Arthrobacter protoformiae* is already known [Applied and Environmental Microbiology, 55, 3107-3112 (1989)], there has been no knowledge regarding the amino acid sequence or gene structure of Endo-A, and hence there is no method of Endo-A production by gene engineering.

The technical problem underlying the present invention is to provide nucleic acid molecules having a nucleotide sequence encoding a polypeptide possessing Endo-A activity, which would allow the provision of recombinant nucleic acid molecules containing such molecules encoding a polypeptide possessing Endo-A activity, for example, expression vectors, which would then allow the production of polypeptides possessing Endo-A activity on an industrial scale using transformants comprising appropriate expression vectors.

This technical problem has been solved by the provision of the embodiments characterized in the claims.

The present invention describes the elucidation of the amino acid sequence of Endo-A and the nucleotide sequence encoding Endo-A using an Endo-A producing bacterial strain (*Arthrobacter protoformiae* AKU 0647). Also described is an advantageous method for industrial scale production of Endo-A using the Endo-A gene.

Thus, in a first aspect, the present invention relates to a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide possessing Endo-A activity or functionally equivalent variants thereof selected from the group consisting of:

- (a) nucleic acid molecules comprising the coding region of the nucleotide sequence as depicted in SEQ ID NO:2, or a fragment thereof;
- (b) nucleic acid molecules encoding the amino acid sequence as depicted in SEQ ID NO:1 or a fragment thereof;
- (c) nucleic acid molecules encoding an amino acid sequence resulting from deletion, addition, insertion or substitution of one or more amino acids in the amino acid sequence of SEQ ID NO:1; and
- (d) nucleic acid molecules capable of hybridizing to any one of the nucleic acid molecules of (a) to (c).

The nucleic acid molecule of the invention may be DNA or RNA. In the case of DNA it is preferably cDNA or genomic DNA. The nucleic acid molecules may, for instance, be produced by recombinant techniques, isolated or chemically synthesized. In a preferred embodiment, the nucleic acid molecules of the present invention are derived from microorganisms, preferably a bacterium and most preferably from bacteria belonging to the genus *Arthrobacter*.

In a most preferred embodiment, the nucleic acid molecule according to the invention is derived from bacteria of the species *Arthrobacter protoformiae*, namely from strain AKU 0647 (deposited as FERM BP-4948).

Furthermore, the present invention relates to recombinant nucleic acid molecules which comprise a nucleic acid molecule according to the invention which encodes an endo- β -N-acetylglucosaminidase A. Such recombinant nucleic acid molecules comprise, i.e. vectors, plasmids, bacteriophages, cosmids etc.

In a preferred embodiment the nucleic acid molecule present in such a recombinant molecule is operably linked to

expression control sequences which allow for expression in prokaryotic or eukaryotic host cells. Such expression control sequences are well known in the art and include, for example, promoters, enhancers, transcription termination signals etc.

In another embodiment, the present invention relates to an expression vector which comprises the nucleic acid molecule of the present invention having a nucleotide sequence encoding a polypeptide possessing Endo-A activity or functionally equivalent variant, wherein the expression vector is capable of propagating in a prokaryotic or eukaryotic cell.

In another embodiment, the present invention relates to a cell of a prokaryote or eukaryote transformed with an expression vector of the present invention.

In still another embodiment, the present invention relates to a method for producing a polypeptide possessing Endo-A activity or functionally equivalent variants thereof, comprising the steps of:

(a) cultivating a transformant obtained by introducing an expression vector into a host cell, the expression vector containing a nucleic acid molecule of the present invention; and

(b) recovering the polypeptide possessing Endo-A activity or functionally equivalent variants thereof from the culture obtained in Step (a).

Furthermore, the present invention relates to the polypeptide having Endo-A activity encoded by the nucleic acid molecules of the present invention or obtainable by the described process.

In another embodiment, the present invention relates to oligonucleotides which specifically hybridize to a nucleic acid molecule according to the invention. Such oligonucleotides have preferably a length of at least 15 nucleotides, more preferably of at least twenty nucleotides. Furthermore, they are preferably not longer than 100, most preferably not longer than 50 nucleotides. The expression that they "specifically hybridize" to the nucleic acid molecules of the present invention means that they show no significant to no cross-hybridization to sequences encoding other proteins.

Preferably, these oligonucleotides show an identity of at least 80%, most preferably of more than 90% and in particular of more than 95% over their whole lengths to corresponding parts of the nucleic acid molecules of the present invention. Such oligonucleotides may be used, for example, for the screening of nucleic acid sequences encoding Endo-A or as PCR primers.

Furthermore, the present invention relates to antibodies which specifically recognize the polypeptides of the present invention as well as to fragments of such antibodies having this property.

The entire amino acid sequence of Endo-A and the nucleotide sequence of the gene encoding the enzyme have first been provided by the present invention, thereby enabling an advantageous, industrial-scale production of a polypeptide possessing Endo-A activity using recombinant DNA technology.

Figure 1 shows a restriction map of the DNA fragment amplified by PCR.

Figure 2 shows restriction maps of 3 kb Cia I insertion fragment and 2.5 kb Hind III/Pst I insertion fragment.

Figure 3 shows the results of western blotting of endo-A.

The term endo- β -N-acetylglucosaminidase A as used herein is defined as possessing the following physico-chemical properties described in Applied and Environmental Microbiology, 55, 3107-3112 (1989):

1. Action

Acts on the N-linked sugar chain of glycoproteins to break the GlcNAc β 1-4GlcNAc bond of di-N-acetylchitobiose at the reduction end of the sugar chain.

2. Substrate specificity

Acts on oligomannose type sugar chains, glycopeptides and glycoproteins but not on complex sugar chains.

3. Optimum pH and pH stability

Optimum pH is between 5.0 and 11.0; the enzyme is stable in the pH range from 5.0 to 7.0.

4. Optimum temperature and temperature stability

Optimum temperature is 60°C; the enzyme is stable up to 60°C.

The term "a polypeptide possessing Endo-A activity" as used herein includes not only native Endo-A but also its variations due to modification of amino acid sequence by, for example, deletion, substitution, insertion, or addition of amino acid residue(s), as long as they retain Endo-A activity.

"Native Endo-A" as used herein includes, but is not limited to, those produced by *Arthrobacter* strains. Also included are those derived from other microorganisms, such as other bacteria, yeasts, Actinomycetes, fungi, Ascomycetes, and Basidiomycetes, and those derived from plants and animal cells.

The term "functionally equivalent variant" as used herein is defined as follows:

A naturally-occurring protein can undergo amino acid deletion, insertion, addition, substitution and other variations in its amino acid sequence due to modifications, etc. of the protein itself in vivo or during purification, as well as due to polymorphism and mutation of the gene encoding it. It is a well-known fact that there are some such polypeptides which

are substantially equivalent to variation-free proteins in terms of physiological or biological activity. A polypeptide structurally different from the corresponding protein, but having no significant functional difference from the protein is referred to as a functionally equivalent variant.

The same applies to polypeptides prepared by artificially introducing such variations into the amino acid sequence of a protein. Although more diverse variants can be thus obtained, the resulting variants are construed as functionally equivalent variants, as long as their physiological activity is substantially equivalent to that of the original variation-free protein.

For example, the methionine residue at the N-terminus of a protein expressed in *Escherichia coli* is reportedly often removed by the action of methionine aminopeptidase, but some such expressed proteins have the methionine residue and others do not. However, the presence or absence of the methionine residue does not affect protein activity in most cases. It is also known that a polypeptide resulting from replacement of a particular cysteine residue with serine in the amino acid sequence of human interleukin 2 (IL-2) retains IL-2 activity [Science, 224, 1431 (1984)].

In addition, in producing a protein by gene engineering, the desired protein is often expressed as a fused protein. For example, the N-terminal peptide chain derived from another protein is added to the N-terminus of the desired protein to enhance the expression of the desired protein, or purification of the desired protein is facilitated by adding an appropriate peptide chain to the N- or C-terminus of the desired protein, expressing the protein, and using a carrier showing affinity for the peptide chain added.

Also, with regards to a codon (triplet base combination) determining a particular amino acid on the gene, 1 to 6 kinds are known to exist for each amino acid. Therefore, there can be a large number of genes encoding an amino acid sequence, though depending on the amino acid sequence. In nature, a gene is not stable, and it is not rare for a gene to undergo nucleic acid variation. A variation on the gene may not affect the amino acid sequence to be encoded (silent variation); in this case, it can be said that a different gene encoding the same amino acid sequence has been generated. The possibility is therefore not negligible that even when a gene encoding a particular amino acid sequence is isolated, a variety of genes encoding the same amino acid sequence are produced after many generations of the organism containing it.

Moreover, it is not difficult to artificially produce a variety of genes encoding the same amino acid sequence by means of various gene engineering techniques.

For example, when a codon used in the natural gene encoding the desired protein is low in availability in the host used to produce the protein by gene engineering, the amount of protein expressed is sometimes insufficient. In this case, expression of the desired protein is enhanced by artificially converting the codon into another one of high availability in the host without changing the amino acid sequence encoded. Thus, it is of course possible to artificially produce a variety of genes encoding a particular amino acid sequence. Such artificially produced different polynucleotides are therefore included in the scope of the present invention, as long as an amino acid sequence disclosed herein is encoded.

Additionally, a polypeptide resulting from at least one change, such as deletion, addition, insertion or substitution, of one or more amino acid residues in the amino acid sequence of the desired protein commonly possesses an activity functionally equivalent to that of the desired protein; nucleic acid molecules encoding such polypeptides are thus also included in the scope of the present invention, whether isolated from natural sources or produced artificially.

In general, nucleotide sequences of nucleic acid molecules encoding functionally equivalent polypeptides often show high homology to each other. Nucleic acid molecules capable of hybridizing to a nucleic acid molecule of the present invention, preferably under stringent conditions, and encoding a polypeptide possessing Endo-A activity, are therefore also included in the scope of the present invention.

The present invention is hereinafter described in detail with reference to Endo-A derived from *Arthrobacter protoformiae* AKU 0647.

The strain *Arthrobacter protoformiae* AKU 0647 has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology of 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan on August 14, 1991 under the Budapest Treaty, as accession number FERM BP-4948.

- 50 1) First, *Arthrobacter protoformiae* AKU 0647 is cultured in accordance with the method described in Applied and Environmental Microbiology, 55, 3107-3112 (1989). Endo-A produced by the *Arthrobacter protoformiae* AKU 0647 is then isolated from the culture and purified.
- 55 2) Second, information regarding a partial amino acid sequence of the purified Endo-A is obtained. The partial amino acid sequence is determined over the region of 10 to 20 residues in the N-terminal amino acid sequence of Endo-A by directly subjecting the purified Endo-A to amino acid sequencing based on Edman degradation by a conventional method (Protein Sequencer 476A, produced by Applied Biosystems). Alternatively, it is effective to conduct amino acid sequencing for a purified peptide fragment obtained by subjecting the purified Endo-A to limited hydrolysis by the action of a protein hydrolase with high specificity, such as Achromobacter protease I or N-tosyl-L-phenylalanylchloromethylketone (TPCK)-trypsin, and separating and purifying the resulting peptide fragments by reversed-phase HPLC.

3) On the basis of the thus-obtained partial amino acid sequence information, the Endo-A gene is cloned. For this purpose, a commonly used PCR or hybridization method is employed.

5 a) On the basis of the partial amino acid sequence information, synthetic oligonucleotides are designed for use as Southern hybridization probes.

b) Separately, the genomic DNA of *Arthrobacter protoformiae* AKU 0647 is completely digested with the appropriate restriction enzymes and subjected to agarose gel electrophoresis, and the resulting fragments are blotted onto a nylon membrane by a conventional method.

10 c) Hybridization of the separated DNA fragments with the synthetic oligonucleotides designed on the basis of the partial amino acid sequence information is conducted under commonly used conditions. For example, the nylon membrane is blocked in a prehybridization solution containing salmon sperm DNA, and each ³²P-labeled synthetic oligonucleotide is added, followed by overnight incubation. After the nylon membrane is washed, an autoradiogram is taken to detect a DNA fragment that hybridizes to the synthetic oligonucleotide probe. The DNA fragment corresponding to the band detected is extracted from the gel and purified.

15 d) The thus-obtained DNA fragment, which hybridizes to the synthetic oligonucleotide probe, is inserted into a plasmid vector by a commonly used method. Useful plasmid vectors include, but are not limited to, pUC18, pUC19, pUC119 and pTV118N.

20 e) The recombinant plasmid is then introduced to a host to transform the host. When the host is *Escherichia coli*, it may be of a wild strain or a variant strain, as long as it is capable of being transformed. This plasmid introduction can be achieved by a commonly used method, such as the method described at page 250 of the Molecular Cloning, A Laboratory Manual (T. Maniatis et al., Cold Spring Harbor Laboratory Press, 1982).

25 f) Next, a transformant harboring the desired DNA fragment is selected.

For this purpose, the characteristics of the plasmid vector are utilized. In the case of pUC19, for instance, colonies having a foreign gene introduced thereto are selected by selecting ampicillin-resistant colonies on an ampicillin-containing plate, or selecting ampicillin-resistant white colonies on a plate containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG).

30 g) The colony having a vector containing the desired DNA fragment is then selected out of the above population. This selection is achieved by using colony hybridization or plaque hybridization, chosen appropriately according to vector types. PCR methods are also applicable.

35 h) Once the vector containing the desired DNA fragment is selected, the nucleotide sequence of the desired DNA fragment inserted in this vector is determined by an ordinary method, such as the dideoxy chain terminator method [Proceedings of the National Academy of Sciences of the USA, 74, 5463 (1977)]. The thus-determined nucleotide sequence is compared with the N-terminal sequence, partial amino acid sequence, molecular weight, etc. of Endo-A, to determine whether the nucleotide is the entire or partial portion of the desired Endo-A gene. From the thus-obtained DNA fragment containing the Endo-A gene, the structure of the Endo-A gene and the entire amino acid sequence of Endo-A are determined.

40 i) When the vector containing the desired DNA fragment does not contain the full-length Endo-A gene; the desired full-length Endo-A gene can be obtained by digesting the genomic DNA of *Arthrobacter protoformiae* AKU 0647 with other restriction enzymes, obtaining the lacking portion from the digests by hybridization, etc. using a part of the DNA fragment obtained above as a probe, as described above, and then joining the lacking portion.

Attempts to clone the Endo-A gene derived from *Arthrobacter protoformiae* AKU 0647 were made in order to obtain the desired gene by carrying out the PCR method using oligonucleotide primers designed on the bases of the following information on partial amino acid sequences, but all failed to clone the desired gene.

In consideration of these facts, extensive investigations have been carried out and it has been found that a portion of the desired Endo-A gene can be amplified by using particular synthetic oligonucleotides designed and synthesized on the basis of an internal partial amino acid sequence of Endo-A as primers for PCR which uses genomic DNA as templates.

50 The present invention is hereinafter described in more detail. First, using synthetic oligonucleotide primers designed on the basis of partial amino acid sequence information and the genomic DNA of the *Arthrobacter protoformiae* AKU 0647 strain as a template, PCR is carried out to yield the desired gene fragment. Specifically, oligonucleotide primer 1 (SEQ ID NO:6) designed from the N-terminal amino acid sequence A-23 (SEQ ID NO:5), oligonucleotide primer 2 (SEQ ID NO:8) designed from the partial amino acid sequence A-46 (SEQ ID NO:7), oligonucleotide primer 3 (SEQ ID NO:10) designed from the partial amino acid sequence A-20 (SEQ ID NO:9), and oligonucleotide primer 4 (SEQ ID NO:12) designed from the partial amino acid sequence A-12 (SEQ ID NO:11), are synthesized. To facilitate the determination of the nucleotide sequence of the amplification product, a BamHI site has been added to the 5' end side of primer 1, and an EcoRI site to the 5' end sides of the other primers.

PCR is conducted in accordance with the method described in "PCR Technology", edited by Erlich H.A., published

by Stockton Press in 1989, using the Gene Amp Reagent Kit (produced by Perkin-Elmer Cetus Instruments), for instance. The reaction is carried out 30 cycles at 94°C for 1 minute, 49°C for 1 minute and 30 seconds and 72°C for 1 minute and 30 seconds each. After a first PCR is conducted with a combination of primers 1 and 2 using the genomic DNA of the *Arthrobacter protoformiae* AKU 0647 strain as a template, a second PCR is conducted with a combination of primers 1 and 3 or a combination of primers 1 and 4 using a portion of the first reaction mixture; subsequent agarose gel electrophoresis analysis of the second reaction mixture fails to detect a clear band attributable to amplified DNA. An additional PCR with a combination of primers 1 and 4 yields a specific band attributable to amplified DNA in agarose gel electrophoresis. The amplified DNA fragments are subjected to base sequencing by a commonly used method, e.g., the dideoxy chain terminator method. A sequence corresponding to a partial amino acid sequence of Endo-A was detected, and a portion of the desired Endo-A gene is successfully obtained. Of course, by conducting an additional procedure of the hybridization method using the thus-obtained gene fragment as a probe, the gene encoding the full-length of Endo-A sequence can be cloned.

The thus-obtained entire nucleotide sequence of the gene for the Endo-A produced by *Arthrobacter protoformiae* AKU 0647 was determined as set forth in SEQ ID NO:2, and the entire amino acid sequence deduced therefrom was determined as set forth in SEQ ID NO:1. It should be noted that there are numerous nucleotide sequences corresponding to SEQ ID NO:1 in addition to the nucleotide sequence of SEQ ID NO:2, and all the nucleic acid molecules having such nucleotide sequences are included in the scope of the present invention. The nucleic acid molecules of the present invention also include the nucleic acid molecules encoding a polypeptide having a portion of the amino acid sequence of SEQ ID NO:1 and still retaining Endo-A activity or functionally equivalent activity. Nucleic acid molecules having a portion of the nucleotide sequence set forth in SEQ ID NO:2 and encoding a polypeptide possessing Endo-A activity or functionally equivalent activity are also included in the scope of the present invention. Also included are nucleic acid molecules capable of hybridizing to the nucleic acid molecules as mentioned above and encoding a polypeptide possessing Endo-A activity or functionally equivalent activity.

Using the entire Endo-A gene whose entire nucleotide sequence has been determined as described above, or a portion thereof, as a probe for hybridization, DNA encoding a polypeptide possessing Endo-A activity and having high homology to the Endo-A gene can be selected from a genomic DNA or cDNA library derived from an organism other than *Arthrobacter protoformiae* AKU 0647. Hybridization can be conducted using commonly used conditions. For example, nylon membranes where the genomic DNA library or cDNA library obtained from an organism other than *Arthrobacter protoformiae* AKU 0647 is blotted are prepared. The nylon membrane is blocked at 65°C in a prehybridization solution containing 6 x SSC, 0.5% SDS, 5 x Denhardt's solution and 100 µg/ml salmon sperm DNA, and each ³²P-labeled synthetic oligonucleotide probe is added, which is followed by overnight incubation at 65°C. After the nylon membrane is washed once with 6 x SSC at room temperature for 10 minutes, and once with 2 x SSC containing 0.1% SDS at room temperature for 10 minutes, and once with 0.2 x SSC containing 0.1% SDS at 45°C for 30 minutes, an autoradiogram is taken to detect DNA fragments that hybridizes to the probe. Genes showing different degrees of homology can be obtained by changing washing and other conditions.

On the other hand, a primer for PCR reaction can be designed from the nucleotide sequence of the gene of the present invention. It is possible to detect a gene fragment highly homologous to the gene of the present invention or obtain the entire gene, by carrying out PCR using this primer.

For producing a polypeptide possessing Endo-A activity using the Endo-A gene of the present invention, the following method is advantageous.

First, a host is transformed with a vector containing the desired Endo-A gene. This transformant is then cultured under commonly used conditions to produce a polypeptide possessing Endo-A activity. As the case may be, the polypeptide is produced in the form of an inclusion body. Useful hosts include microorganisms, animal cells and plant cells.

It is advantageous to confirm expression by, for example, determining Endo-A activity. Activity can be determined by the method described in Applied and Environmental Microbiology, 55, 3107-3112(1989), using a recombinant *Escherichia coli* cell extract as an enzyme solution.

When the desired expression of Endo-A is noted, Endo-A can be efficiently produced by setting optimum conditions for Endo-A expression as to medium composition, medium pH, culturing temperature, amount of inducer used, timing of induction, culturing time, etc., in cases where the transformant is *Escherichia coli*.

Endo-A can be purified from the transformant culture by an ordinary method. The transformant, like *Escherichia coli*, intracellularly accumulates Endo-A during cultivation. The cultivated transformant cells are collected by centrifugation, disrupted by ultrasonication, or the like, and then subjected to centrifugation, etc. to yield a cell-free extract, which can be purified by common protein purification methods, such as salting-out and various chromatographies including ion exchange, gel filtration, hydrophobic and affinity chromatographies. Depending on the host-vector system used, the expression product is extracellularly secreted by the transformant; in this case, the product can be purified from the culture supernatant in the same manner as that described above.

When Endo-A is intracellularly produced by the transformant, various enzymes are also present in the cell, but purification of the Endo-A is very easy, because such enzymes are present in trace amounts, relative to the amount of

Endo-A. When Endo-A is extracellularly secreted, medium components, etc. are also present. However, these co-present substances normally contain almost no protein components that can interfere with Endo-A purification; this is advantageous in that there is no need for the painstaking separation procedures for purification of Endo-A from the *Arthrobacter protoformiae* AKU 0647 culture.

When the host is *Escherichia coli*, the expression product is sometimes formed as an insoluble inclusion body. In this case, cells are collected by centrifugation after cultivation, disrupted by ultrasonication, or the like, then subjected to centrifugation, etc. to separate the insoluble fraction containing the inclusion body. After being washed, the inclusion bodies are solubilized with a commonly used protein solubilizer, such as urea or guanidine hydrochloride, followed by purification by various chromatographies, such as ion exchange, gel filtration, hydrophobic and affinity chromatographies, as necessary, after which a refolding treatment by dialysis or dilution is conducted to yield the desired polypeptide retaining Endo-A activity. This standard preparation may be purified by various chromatographies to yield a highly pure polypeptide possessing Endo-A activity.

The same procedures as those described above may be used for producing and purifying a functionally equivalent variant of the DNA mentioned above.

As described above, the present invention provides the primary structure of Endo-A produced by *Arthrobacter protoformiae* AKU 0647, and the gene structure thereof. The elucidation of the gene structure of the present invention permits the biotechnological production of a polypeptide possessing Endo-A activity or functionally equivalent variant thereof. By the use of the present method using recombinant DNA technology, a highly pure polypeptide possessing Endo-A activity or a functionally equivalent variant thereof can be produced at low cost.

EXAMPLES

The following examples illustrate the present invention.

Example 1. Cloning of Endo-A structural gene

(1) Extraction and purification of genomic DNA

Arthrobacter protoformiae AKU 0647 (FERM BP-4948), an Endo-A producer, was inoculated to 10 ml of a medium containing 0.5% yeast extract, 0.5% peptone and 0.5% NaCl, pH 7.5, and pre-cultured at 28°C for 18 hours, after which 10 ml of the culture broth was transferred to each of five conical flasks each containing 100 ml of the same medium as above, and subjected to shaking culture for 24 hours. After completion of the cultivation, the culture broth was centrifuged to collect cells, which were then twice washed with a saline-EDTA solution (0.15 M NaCl, 0.1 M EDTA, pH 8.0) and suspended in 20 ml of a saline-EDTA solution, after which 0.5 ml of a lysozyme solution [dissolved at a concentration of 20 mg/ml in a saline-TE solution (0.1 M NaCl, 10 mM EDTA, 0.1 M Tris-HCl, pH 8.0)] was added, followed by shaking at 37°C for 10 minutes. Subsequently, 5 ml of a 5% SDS solution (dissolved in saline-TE solution) was added. After the mixture was shaken at 60°C for 20 minutes, 130 µl of proteinase K (10 mg/ml) was added (final concentration 50 µg/ml), followed by incubation at 37°C for 3 hours. The reaction mixture was then cooled to room temperature and gently stirred in the presence of an equal volume of phenol saturated with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After centrifugation at 8000 rpm for 20 minutes, the upper layer was collected (hereinafter referred to as phenol extraction). To the water layer, a 2-fold volume of cold ethanol was gradually added to precipitate DNA, which was then wound around a glass rod, washed with 70%, 80% and 90% cold ethanol solutions, and gently dried in air (hereinafter referred to as ethanol precipitation). This dry product was dissolved in 18 ml of 0.1 x SSC [20 x SSC (3 M NaCl, 0.3 M sodium citrate) used after dilution]; 2 ml of 10 x SSC and 100 µl of RNase A (10 mg/ml) (final concentration 50 µg/ml) were added, followed by incubation at 37°C for 1 hour. After completion of the reaction, deproteinization and ethanol precipitation were conducted. The resulting precipitate was dissolved in 2 ml of 0.1 x SSC and dialyzed against TE buffer for 24 hours, then subjected to phenol/chloroform extraction, chloroform extraction and ethanol precipitation, followed by centrifugation to collect DNA, which was then dissolved in TE buffer to yield a genomic DNA solution. The concentration of the genomic DNA thus obtained was determined from its absorbance to be 509 µg/ml. Agarose electrophoresis demonstrated that the genomic DNA had a size not shorter than 24 kb.

(2) Determination of partial amino acid sequence of Endo-A

Endo-A as purified by the method described in Applied and Environmental Microbiology, 55, 3107-3112 (1989) was directly subjected to amino acid sequencing by gas phase Edman degradation to determine the N-terminal amino acid sequence A-23 (SEQ ID NO:5). After being pyridylethylated [1 nmol of endo-A protein was applied to a desalting column (Fast Desalting Column PC3.2/10, Pharmacia), previously equilibrated with 450 mM N-ethylmorpholine/formate buffer, pH 8.5, and eluted with the same buffer; the resulting eluate was collected in a glass vial and concentrated to dryness; separately, 10 µl of pyridine, 2 µl of 4-vinylpyridine, 2 µl of tri-N-butylphosphine and 10 µl of water were placed in a glass test tube larger in diameter than the vial; the sample-containing glass vial was placed in this glass test tube; after the glass test tube was sealed, a reaction was carried out at 100°C for 5 minutes;

after completion of the reaction, the glass vial was taken out from the test tube and thoroughly dried; the resulting pyridylethylated product was used for lysylendopeptidase digestion], the enzyme protein was digested with lysylendopeptidase [40 µl of a 10 mM Tris-HCl buffer (pH 7.5) containing 4 M urea, 50 µl of a 10 mM Tris-HCl buffer (pH 7.5), 10 µl of 0.1 M calcium chloride, and 2 pmol of lysylendopeptidase were added to the glass vial, followed by overnight reaction at 37°C]; from the resulting digest, a peptide fragment was separated and purified by HPLC (Smart System, produced by Pharmacia; column, mRPC C2/C18, SC2.1/10; flow rate, 1 ml/min; eluent A, 0.1% trifluoroacetic acid solution; eluent B, acetonitrile containing 0.1% trifluoroacetic acid; elution was conducted on a density gradient from 0% of eluent B at the time of sample application to 10% of eluent B at the time of completion of sample application, after which the eluent B concentration was increased to 60% over an 85-minute period). Each peptide fraction was subjected to amino acid sequencing to determine the partial amino acid sequences A-46 (SEQ ID NO:7), A-20 (SEQ ID NO:9), and A-12 (SEQ ID NO:11).

(3) Preparation of gene library of *Arthrobacter protoformiae* AKU 0647 strain

To 10 µl of the genomic DNA (509 µg/ml) prepared in (1) above, 8 units of the restriction enzyme Sau3AI (produced by Takara Shuzo) were added to make a total volume of 50 µl, after which the genomic DNA was digested at 37°C for 20, 30, 40 and 60 seconds. The reaction was terminated at each time point by adding 15 µl of 100 mM EDTA (pH 8.0) and heating at 60°C for 20 minutes.

Agarose gel electrophoresis demonstrated that this genomic DNA was partially digested from about 24 kb to about 1 kb with the progress of the reaction.

The above partial digest solutions were combined and subjected to agarose electrophoresis; DNA fragments of about 4 to 23 kb size were cut out, followed by DNA recovery using the EASY TRAP (produced by Takara Shuzo) and subsequent ethanol precipitation; the resulting precipitate was dissolved in 10 µl of TE buffer.

Using a ligation kit (produced by Takara Shuzo), the λEMBL3 arm (produced by STRATAGENE) and each of the about 4 to 23 kb DNA fragments obtained were allowed to react at 16°C for 10 minutes in the composition shown in Table 1 to yield a recombination vector.

Table 1

λEMBL3 arm	0.5µL (0.5µg)
DNA fragment	8.5µL
3M NaCl	1.0µL
Solution B (in kit)	10 µL
Total	20 µL

This reaction mixture was subjected to ethanol precipitation; the resulting precipitate was dissolved in 4 µl of TE buffer to yield a ligation DNA solution, which was then subjected to in vitro packaging using the Gigapack II Gold Packaging Extract (produced by STRATAGENE).

The phage liquid prepared by the in vitro packaging, 1, 5 or 10 µl each, was added to 600 µl of an *E. coli* P2392 suspension [prepared by culturing the strain in 50 ml of TB medium (0.5% NaCl, 1.0% peptone, pH 7.4) containing 10 mM MgSO₄ and 0.2% maltose at 28°C for 10 hours, collecting cells, and suspending the cells in 10 mM MgSO₄ to an absorbance (600 nm) of 0.5], followed by incubation at 37°C for 15 minutes, to infect the strain with the phage.

Next, to 3 ml of top agar [NZY medium (0.5% NaCl, 0.5% yeast extract, 0.2% MgSO₄ · 7H₂O, 1.0% NZ amine, 0.7% agarose], previously incubated at 50°C, the above phage-infected liquid was added, followed by immediate mixing, after which the mixture was poured over bottom agar (NZY medium, 3% agar), previously incubated at 37°C, followed by incubation at 37°C for 8 hours. After confirming that the plaques on the plate became 0.5 to 1.0 mm in size, they were stored in a refrigerator (4°C).

The plaques appeared on the medium was used as a gene library. Out of the resulting plaques, 6 were randomly picked up and examined for DNA inserts. Four of the 6 clones were found to contain an about 10 kb DNA insert.

Also, the same procedure was carried out to yield a gene library consisting of about 10000 clones.

(4) Cloning of DNA fragment containing Endo-A gene

Primer 1 (SEQ ID NO:6), designed from the N-terminal amino acid sequence A-23 (SEQ ID NO:5) determined in (2) above, primer 2 (SEQ ID NO:8), designed from the partial amino acid sequence A-46 (SEQ ID NO:7), primer 3 (SEQ ID NO:10), designed from the partial amino acid sequence A-20 (SEQ ID NO:9), and primer 4 (SEQ ID NO:12), designed from the partial amino acid sequence A-12 (SEQ ID NO:11), were synthesized. To facilitate the determination of the nucleotide sequence of the amplification product, a BamHI site has been added to the 5' end

side of primer 1, and an EcoRI site to the 5' end side of the other primers.

Using these primers, PCR was conducted with the genomic DNA of the *Arthrobacter protoformiae* AKU 0647 strain as a template, in accordance with the method described in "PCR Technology", edited by Erlich H.A., published by Stockton Press in 1989, using the Gene Amp Reagent Kit (produced by Perkin-Elmer Cetus Instruments). The reaction was conducted 30 cycles at 94°C for 1 minute, 49°C for 1 minute and 30 seconds and 72°C for 1 minute and 30 seconds each.

This PCR resulted in specific amplification of a DNA fragment with a combination of primer 1 (SEQ ID NO:6) and primer 4 (SEQ ID NO:12) in a single operation.

The DNA fragment (about 1.2 kb) amplified with the combination of primers 1 and 4 was cut out, followed by DNA collection using the EASY TRAP (produced by Takara Shuzo). This DNA fragment was digested with the restriction enzymes EcoRI and BamHI (both produced by Takara Shuzo) and ligated at the EcoRI and BamHI sites of the plasmid pBluescript (produced by STRATAGENE) using a ligation kit (produced by Takara Shuzo).

To draw the restriction map for the amplified DNA fragment (about 1.2 kb), the fragment was digested with the restriction enzyme HindIII (produced by Takara Shuzo), which revealed that one HindIII site was present near the center of the DNA fragment (Figure 1).

Next, this amplified DNA fragment was analyzed by the dideoxy chain terminator method to determine the nucleotide sequences from both the BamHI and EcoRI sites. In addition, the nucleotide sequences of both sides of the only HindIII site at the center of this amplified DNA fragment were also determined by the dideoxy chain terminator method. The nucleotide sequence on the BamHI site side is shown in SEQ ID NO:13 in the sequence listing; the nucleotide sequence on the EcoRI site side is shown in SEQ ID NO:14 in the sequence listing; the nucleotide sequence on the BamHI site side of the HindIII site is shown in SEQ ID NO:15 in the sequence listing; the nucleotide sequence on the EcoRI site side of the HindIII site is shown in SEQ ID NO:16 in the sequence listing.

As a result, in addition to the sequences of primers 1 and 4, a sequence corresponding to a partial amino acid sequence of Endo-A was found in the sequence determined; a portion of the desired Endo-A gene was successfully obtained.

(5) Cloning of Endo-A gene

Next, using the DNA fragment (about 1.2 kb) obtained in (4) above as a probe, the gene library prepared in (3) above was screened.

First, 480 µg of the amplified DNA fragment (about 1.2 kb) was labeled using the ECL random prime labeling system (produced by Amersham Corporation), as directed in the system protocol.

Using this labeled DNA fragment as a probe, plaque hybridization with the gene library prepared in (3) above was carried out. Plaque hybridization was conducted by the method described in the instruction manual for the ECL random prime labelling system and the method described in "Molecular Cloning - A Laboratory Manual", 2nd edition, edited by Maniatis et al., Chapter 2, pp. 108-122, published by Cold Spring Harbor Laboratory Press in 1989. Specifically, a nylon membrane produced by Amersham Corporation (trade name Hybond-N+) was cut into plate pieces and marked with an about 1 mm groove to identify the nylon membrane orientation, and placed on a plate of the gene library prepared in (3) above. This plate was kept standing for 5 minutes, after which the nylon membrane was slowly peeled from the plate, placed on filter paper, moistened with 0.5 M NaOH, with face contacting the plate up, and kept standing for 5 minutes. This nylon membrane was then transferred onto dry filter paper to remove the water. The DNA was immobilized onto a nylon membrane using FUNA-UV-1-LINKER FS-800 (produced by Funakoshi). A filter for plaque hybridization was thus prepared.

The filter thus prepared was subjected to prehybridization in a solution containing 5 x SSC [1 x SSC = solution of 8.77 g of NaCl and 4.41 g of sodium citrate in 1 l of water], 0.5% SDS, 100 µg/ml salmon sperm DNA and 5 x Denhardt's (containing bovine serum albumin, polyvinylpyrrolidone and Ficoll each at 0.1% concentration) at 60°C for 1 hour, after which the DNA fragment labeled as above, as a labeled probe, was added to make a concentration of 5 ng/ml (the labeled probe was previously heated in boiling water for 5 minutes, then rapidly quenched in ice), followed by hybridization at 60°C for 8 hours and 50 minutes.

Next, the filter was sequentially washed in 1 x SSC containing 0.1% SDS at 60°C for 15 minutes, in 0.5 x SSC containing 0.1% SDS at 60°C for 15 minutes, and in buffer A (0.1 M Tris-HCl, pH 7.5, 0.6 M NaCl) at 25°C for 1 minute. Next, to further diminish the hybridization background, the plate was washed in the liquid block attached to the system diluted 20 times with buffer A at 25°C for 30 minutes.

Next, an antibody reaction was conducted in a solution containing the HRP-labeled anti-fluorescein antibody attached to the system in a 1/1000 volume of buffer A (containing 0.5% BSA) at 25°C for 30 minutes. Next, the plate was washed in buffer A containing 0.5% BSA at 25°C for 30 minutes and in buffer A containing 0.1% Tween 20 at 25°C for 10 minutes. The same procedure was carried out 3 times in total.

Next, in a solution consisting of a 1:1 mixture of the detection reagents 1 and 2 attached to the system, a detection reaction was carried out at 25°C for 1 minute, after which this filter was exposed to light for 20 minutes in the same manner as that for autoradiography.

As a result, 13 positive plaques were obtained, each of which was suspended in 500 µl of SM buffer (0.58%

NaCl, 0.2% MgSO₄ · 7H₂O, 50 mM Tris-HCl, pH 7.5, 0.01% gelatin), then kept standing at room temperature for 1 hour, followed by centrifugation. The resulting supernatant was collected as a phage liquid and stored at 4°C after adding one drop of chloroform as a preservative.

5 Phage DNA was collected from the thus-obtained phage liquid. Using this phage DNA as a template, PCR was conducted with primer 1 (SEQ ID NO:6) and primer 4 (SEQ ID NO:12) under the conditions described in (4) above. As a result, 2 of the 13 DNA clones were confirmed to contain the expected about 1.2 kb DNA fragment by agarose gel electrophoresis.

10 To purify the two phage DNAs, the phage liquid prepared above, corresponding to this phage DNA, 1, 5 or 10 µl each, was added to 600 µl of an *E. coli* P2392 suspension [prepared by culturing the strain in 50 ml of TB medium (0.5% NaCl, 1.0% peptone, pH 7.4) containing 10 mM MgSO₄ and 0.2% maltose at 28°C for 10 hours, collecting cells, and suspending the cells in 10 mM MgSO₄ to an absorbance (600 nm) of 0.5], followed by incubation at 37°C for 15 minutes, to infect the strain with the phage liquid.

15 Next, to 3 ml of top agar [NZY medium (0.5% NaCl, 0.5% yeast extract, 0.2% MgSO₄ · 7H₂O, 1.0% NZ amine), 0.7% agarose], previously heated at 50°C, the above phage-infected liquid was added, followed by immediate mixing, after which the mixture was poured onto bottom agar (NZY medium, 3% agar), previously heated at 37°C, followed by incubation at 37°C for 8 hours.

20 The plates on which single plaques appeared were each subjected to plaque hybridization under the same conditions as those described above. From the thus-obtained positive plaques, 2 plates per plaque were selected, and phage liquids were prepared in the same manner as above to yield phage DNA. Using each phage DNA as a template, PCR was conducted under the conditions shown in (4) above with primer 1 (SEQ ID NO:6) and primer 4 (SEQ ID NO:12); 3 of the 4 phage DNAs were confirmed to contain the expected about 1.2 kb DNA fragment by agarose gel electrophoresis.

25 To determine whether or not the phage DNAs obtained are identical, each was digested with the restriction enzymes BamHI and HindIII (both produced by Takara Shuzo); the same electrophoresis pattern was obtained from the two, while a different pattern was obtained from the other one.

A part of the desired Endo-A gene was thus successfully cloned. Of the two phage DNAs obtained (phage DNA 1 and phage DNA 10), phage DNA 1 was used for the following experiments, in view of simplicity in handling.

(6) Subcloning of Endo-A gene

30 DNA clone 1, obtained in (5) above, was digested with each of the restriction enzymes Clal, HindIII, PstI and Sall (all produced by Takara Shuzo) and subjected to agarose gel electrophoresis, after which hybridization was conducted at 60°C for 12 hours by the method described in "Molecular Cloning - A Laboratory Manual -, 2nd edition, edited by Maniatis et al., Chapter 9, pp. 31-58, published by Cold Spring Harbor Laboratory Press in 1989," using the DNA fragment (about 1.2 kb) labeled in (5) above as a probe.

35 As a result, the about 3 kb DNA fragment obtained by digestion with the restriction enzyme Clal was hybridized to the DNA fragment (about 1.2 kb) labeled in (5) above. This about 3 kb DNA fragment showing hybridization was recovered and ligated to the Clal site of pBluescript SK(-) (produced by STRATAGENE). This plasmid was designated as Clal-3kb. The restriction enzyme map for the inserts of Clal-3kb is shown in Figure 2.

40 The nucleotide sequence of the insert in this plasmid was determined by the dideoxy chain terminator method. Although the sequences of primer 1 encoding N-terminal region and primer 4 were found in the insert but only an about 0.3 kb portion from the end of primer 4 in the direction to the C-terminus-coding region was contained. To obtain a DNA fragment encoding the entire C-terminal region, a HindIII-Clal fragment (about 0.9 kb), the insert closest to the C-terminus-coding region in Clal-3kb was labeled in the same manner as in the method described in (5) above. Using this labeled fragment as a probe, DNA clone 1, obtained in (5) above, was digested with each of the restriction enzymes HindIII, KpnI, PstI, Pvull, HindIII-KpnI, HindIII-PstI and HindIII-Pvull and subjected to agarose gel electrophoresis, after which hybridization was conducted at 60°C for 12 hours by the method described in "Molecular Cloning - A Laboratory Manual -, 2nd edition, edited by Maniatis et al., Chapter 9, pp. 31-58, published by Cold Spring Harbor Laboratory Press in 1989."

45 As a result, the about 2.5 kb DNA fragment obtained by digestion with HindIII-PstI was hybridized to this probe. This about 2.5 kb DNA fragment showing hybridization was collected and ligated to the HindIII-PstI site of pBlue-script SK(-) (produced by STRATAGENE). This plasmid was designated as HindIII/PstI-2.5kb. The restriction map for the inserts in HindIII/PstI-2.5kb is shown in Figure 2.

50 The nucleotide sequence of the insert in this plasmid was determined by the dideoxy chain terminator method; the sequence determined was found to contain the HindIII-Clal fragment (about 0.9 kb) from Clal-3kb, with a termination codon on the 3' side.

55 By combining the plasmids HindIII/PstI-2.5kb and Clal-3kb, the full length of the Endo-A gene can be known. An example nucleotide sequence of the open reading frame (ORF) for Endo-A is shown in SEQ ID NO:4 in the sequence listing; the amino acid sequence encoded by that nucleotide sequence is shown in SEQ ID NO:3 in the sequence listing. Also, on the basis of the finding regarding the N-terminal amino acid sequence A-23 (SEQ ID NO:5) of Endo-A obtained in (2) above, an example of nucleotide sequence encoding Endo-A is shown in SEQ ID

NO:2 in the sequence listing; the amino acid sequence encoded by that nucleotide sequence is shown in SEQ ID NO:1 in the sequence listing.

Example 2. Construction of Endo-A expression plasmid

5 (1) Construction of plasmid containing full-length Endo-A gene

The HindIII-Clal fragment in the plasmid HindIII/PstI-2.5kb was replaced with the insert in the plasmid Clal-3kb to yield the Clal-PstI plasmid, which contains the gene encoding Endo-A in full length. The plasmid thus obtained, which contains the full-length Endo-A gene, was designated as pEACP.

10 The *E. coli* XL1-Blue strain transformed with pEACP is referred to as *Escherichia coli* XL1-Blue/pEACP. The *E. coli* XL1-Blue strain transformed with pEACP, with the designation *Escherichia coli* XL1-Blue/pEACP, has been deposited at the National Institute for Bioscience and Human-Technology, Agency of Industrial Science and Technology of 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan on October 5, 1995 under the Budapest Treaty, as accession number FERM BP-5581.

15 (2) Determination of Endo-A activity

10 The *Escherichia coli* XL1-Blue/pEACP was cultured at 37°C for about 10 hours in 5 ml of 2xTY medium containing 100 µg/ml ampicillin. A portion of this culture broth was centrifuged; the resulting supernatant, as a crude enzyme solution, was subjected to Endo-A activity determination by the method described in Applied and Environmental Microbiology, 55, 3107-3112 (1989). Specifically, after the reaction was carried out at 37°C for 1 hour with the composition shown in Table 2, an Endo-A activity of about 9 mU/ml was observed.

Table 2

8mM	Dansylated asparagine glycopeptide	5 µL
200mM	Acetate buffer 8pH 6.0)	10 µL
	Crude enzyme solution	5 µL
	Total	20 µL
	Reaction stopper	5 µL

35 (3) Western blotting of Endo-A

To determine whether or not the Endo-A in the crude Endo-A solution prepared from *Escherichia coli* XL1-Blue/pEACP in (2) above is identical with the endo-A from the *Arthrobacter protoformiae* AKU 0647 strain, western blotting was conducted by the method described in "Molecular Cloning - A Laboratory Manual -, 2nd edition, edited by Maniatis et al., Chapter 18, pp. 60-74, published by Cold Spring Harbor Laboratory Press in 1989." The Endo-A antibody used was prepared by the method described in "Molecular Cloning - A Laboratory Manual -, 2nd edition, edited by Maniatis et al., Chapter 18, pp. 3-17, published by Cold Spring Harbor Laboratory Press in 1989," using Endo-A purified by the method described in Applied and Environmental Microbiology 55, 3107-3112 (1989). The results are shown in Figure 3, in which lane 1 shows the results obtained using about 15 ng of the Endo-A prepared from the *Arthrobacter protoformiae* AKU 0647 strain, lane 2 shows the results obtained using about 2 µg of the protein of the crude enzyme solution prepared from *Escherichia coli* XL1-Blue/pEACP in (2) above.

40 As seen from Figure 3, the Endo-A of *Escherichia coli* XL1-Blue/pEACP was confirmed to be identical with the Endo-A from the *Arthrobacter protoformiae* AKU 0647 strain.

45 Other modifications of the above described embodiments of the invention which are obvious to those skilled in the art are intended to be within the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 16

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 621 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ser	Thr	Tyr	Asn	Gly	Pro	Leu	Ser	Ser	His	Trp	Phe	Pro	Glu	Glu
1					5			10					15	
Leu	Ala	Gln	Trp	Glu	Pro	Asp	Ser	Asp	Pro	Asp	Ala	Pro	Phe	Asn
					20			25					30	
Arg	Ser	His	Val	Pro	Leu	Glu	Pro	Gly	Arg	Val	Ala	Asn	Arg	Val
					35			40					45	
Asn	Ala	Asn	Ala	Asp	Lys	Asp	Ala	His	Leu	Val	Ser	Leu	Ser	Ala
					50			55					60	
Leu	Asn	Arg	His	Thr	Ser	Gly	Val	Pro	Ser	Gln	Gly	Ala	Pro	Val
					65			70					75	
Phe	Tyr	Glu	Asn	Thr	Phe	Ser	Tyr	Trp	His	Tyr	Thr	Asp	Leu	Met
					80			85					90	
Val	Tyr	Trp	Ala	Gly	Ser	Ala	Gly	Glu	Gly	Ile	Ile	Val	Pro	Pro
					95			100					105	
Ser	Ala	Asp	Val	Ile	Asp	Ala	Ser	His	Arg	Asn	Gly	Val	Pro	Ile
					110			115					120	
Leu	Gly	Asn	Val	Phe	Phe	Pro	Pro	Thr	Val	Tyr	Gly	Gly	Gln	Leu
					125			130					135	
Glu	Trp	Leu	Glu	Gln	Met	Leu	Glu	Gln	Glu	Glu	Asp	Gly	Ser	Phe
					140			145					150	
Pro	Leu	Ala	Asp	Lys	Leu	Leu	Glu	Val	Ala	Asp	Tyr	Tyr	Gly	Phe
					155			160					165	
Asp	Gly	Trp	Phe	Ile	Asn	Gln	Glu	Thr	Glu	Gly	Ala	Asp	Glu	Gly
					170			175					180	
Thr	Ala	Glu	Ala	Met	Gln	Ala	Phe	Leu	Val	Tyr	Leu	Gln	Glu	Gln
					185			190					195	
Lys	Pro	Glu	Gly	Met	His	Ile	Met	Trp	Tyr	Asp	Ser	Met	Ile	Asp
					200			205					210	
Thr	Gly	Ala	Ile	Ala	Trp	Gln	Asn	His	Leu	Thr	Asp	Arg	Asn	Lys
					215			220					225	
Met	Tyr	Leu	Gln	Asn	Gly	Ser	Thr	Arg	Val	Ala	Asp	Ser	Met	Phe
					230			235					240	
Leu	Asn	Phe	Trp	Trp	Arg	Asp	Gln	Arg	Gln	Ser	Asn	Glu	Leu	Ala
					245			250					255	
Gln	Ala	Leu	Gly	Arg	Ser	Pro	Tyr	Asp	Leu	Tyr	Ala	Gly	Val	Asp
					260			265					270	

	Val Glu Ala Arg Gly Thr Ser Thr Pro Val Gln Trp Glu Gly Leu		
	275	280	285
5	Phe Pro Glu Gly Glu Lys Ala His Thr Ser Leu Gly Leu Tyr Arg		
	290	295	300
	Pro Asp Trp Ala Phe Gln Ser Ser Glu Thr Met Glu Ala Phe Tyr		
	305	310	315
	Glu Lys Glu Leu Gln Phe Trp Val Gly Ser Thr Gly Asn Pro Ala		
	320	325	330
10	Glu Thr Asp Gly Gln Ser Asn Trp Pro Gly Met Ala His Trp Phe		
	335	340	345
	Pro Ala Lys Ser Thr Ala Thr Ser Val Pro Phe Val Thr His Phe		
	350	355	360
	Asn Thr Gly Ser Gly Ala Gln Phe Ser Ala Glu Gly Lys Thr Val		
	365	370	375
15	Ser Glu Gln Glu Trp Asn Asn Arg Ser Leu Gln Asp Val Leu Pro		
	380	385	390
	Thr Trp Arg Trp Ile Gln His Gly Gly Asp Leu Glu Ala Thr Phe		
	395	400	405
20	Ser Trp Glu Glu Ala Phe Glu Gly Gly Ser Ser Leu Gln Trp His		
	410	415	420
	Gly Ser Leu Ala Glu Gly Glu His Ala Gln Ile Glu Leu Tyr Gln		
	425	430	435
	Thr Glu Leu Pro Ile Ser Glu Gly Thr Ser Leu Thr Trp Thr Phe		
	440	445	450
25	Lys Ser Glu His Gly Asn Asp Leu Asn Val Gly Phe Arg Leu Asp		
	455	460	465
	Gly Glu Glu Asp Phe Arg Tyr Val Glu Gly Glu Gln Arg Glu Ser		
	470	475	480
	Ile Asn Gly Trp Thr Gln Trp Thr Leu Pro Leu Asp Ala Phe Ala		
	485	490	495
30	Gly Gln Thr Ile Thr Gly Leu Ala Phe Ala Ala Glu Gly Asn Glu		
	500	505	510
	Thr Gly Leu Ala Glu Phe Tyr Ile Gly Gln Leu Ala Val Gly Ala		
	515	520	525
	Asp Ser Glu Lys Pro Ala Ala Pro Asn Val Asn Val Arg Gln Tyr		
	530	535	540
35	Asp Pro Asp Pro Ser Gly Ile Gln Leu Val Trp Glu Lys Gln Ser		
	545	550	555
	Asn Val His His Tyr Arg Val Tyr Lys Glu Thr Lys His Gly Lys		
	560	565	570
	Glu Leu Ile Gly Thr Ser Ala Gly Asp Arg Ile Tyr Leu Glu Gly		
	575	580	585
40	Leu Val Glu Glu Ser Lys Gln Asn Asp Val Arg Leu His Ile Glu		
	590	595	600
	Ala Leu Ser Glu Thr Phe Val Pro Ser Asp Ala Arg Met Ile Asp		
	605	610	615
45	Ile Lys Ser Gly Ser Phe		
	620		

(2) INFORMATION FOR SEQ ID NO:2:

- 50 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1863 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Arthrobacter protoformiae*
(B) STRAIN: AKU 0647

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

10	TCTACGTACA ACAGGCCCGCT GTCCTCCCAT TGGTTTCCAG AGGAACATTGC CCAATGGGAA	60
	CCAGACAGTG ATCCAGACGC ACCCTTAAC AGAACGCATG TTCCGCTGGA ACCAGGCCGC	120
15	GTTGCGAATA GGGTAAATGC TAATGCAGAC AAGGACGCAC ACCTTGTTC GTTGTCCGCG	180
	CTAAACAGGC ATACATCAGG TGTTCCATCG CAAGGAGCGC CAGTTTCTA TGAAAATACG	240
20	TTCAGCTATT GGCATTATAAC AGATTTGATG GTTTATTGGG CTGGTTCAAGC TGGCGAAGGC	300
	ATTATCGTTC CGCCAAGTGC CGATGTCATT GATGCATCGC ACCGAAATGG GGTGCCGATT	360
25	TTAGGAAATG TGTTCTTCCC GCCGACGGTT TATGGAGGGC AGCTAGAGTG GCTAGAACAA	420
	ATGTTAGAGC AAGAGGAGGA CGGTTCATTC CCCCTTGCTG ACAAAATTGCT AGAAGTCGCA	480
30	GACTATTATG GGTTTGACGG CTGGTTTATT AACCAAGAAA CAGAAGGGGC AGACGAAGGA	540
	ACAGCCGAAG CCATGCAAGC TTTTCTCGTT TATTTGCAGG AACAAAAGCC AGAAGGCATG	600
35	CACATCATGT GGTATGACTC GATGATTGAT ACAGGGCGA TCGCCTGGCA AAACCATTAA	660
	ACGGATCGAA ATAAAATGTA CTTGCAAAAT GGCTCGACCC GCGTCGCTGA CAGCATTTT	720
40	TTGAACTTTT GGTCGCGTGA CCAGGCCAA TCGAACGAAT TGGCACAAAGC ACTTGGCAGG	780
	TCTCCGTATG ACCTCTATGC CGGAGTGGAT GTGGAAGCAC GAGGGACAAG TACCCCTGTT	840
45	CAGTGGGAAG GCCTGTTTCC TGAAGGAGAA AAGGCGATA CATCACTCGG GTTATACCGT	900
	CCAGATTGGG CATTTCAGTC AAGTGAACAA ATGGAAGCGT TTTATGAAAA AGAACTACAA	960
50	TTTTGGGTTG GCTCGACAGG AAATCCAGCC GAAACAGACG GCCAGTCAAA TTGGCCTGGC	1020
	ATGGCGCACT GGTTTCCCGC GAAAAGCACC GCTACTTCGG TACCCTTGT GACTCACTTT	1080
	AATAACCGCA GCGGCGCTCA GTTTCCGGCA GAAGGCAAAA CTGTGTCGGA ACAGGAATGG	1140
	AATAACCGCA GCCTTCAAGA TGTGCTGCCG ACATGGCGCT GGATTCAGCA TGGCGGCAT	1200
	TTAGAGGCAA CATTTCCTTG GGAAGAAGCG TTTGAAGGGG GAAGCTCGTT ACAATGGCAT	1260
	GGCTCATTAG CGGAAGGAGA ACACGCCAA ATCGAGCTCT ATCAAACAGA GTTGCCGATA	1320
	AGCGAAGGCA CTTCGCTAAC GTGGACATTT AAAAGCGAGC ACGGCAACGA TTTAAATGTG	1380

GGCTTCGTT TAGATGGGGA AGAGGACTTC CGTTATGTGG AAGGAGAACAA GCGTGAATCG 1440
 ATAAATGGTT GGACGCAGTG GACGTTGCCG CTGGATGCGT TTGCTGGTCA GACCGATAACA 1500
 5 GGGCTGGCAT TTGCAGCGGA AGGGAATGAG ACTGGGCTGG CAGAATTCTA TATTGGACAA 1560
 CTGGCCGTAG GTGCTGATAG CGAAAAGCCT GCCGCTCCAA ACGTGAACGT ACGCCAGTAC 1620
 10 GACCCAGACC CGAGTGGCAT TCAGCTCGTA TGGGAAAAAC AAAGCAACGT CCACCATTAC 1680
 CGCGTTTATA AAGAAACAAA GCACGGAAA GAGCTAATTG GCACATCTGC TGGAGATCGA 1740
 ATTTACCTAG AAGGCCTAGT CGAGGAAAGC AAACAAAACG ACGTGCCTCT GCATATAGAA 1800
 15 GCACTAAGTG AAACATTGT GCCAAGTGAT GCTCGCATGA TCGACATAAA AAGCGGCTCG 1860
 TTT 1863

(2) INFORMATION FOR SEQ ID NO:3:

- 20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 645 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

30 Leu Arg Lys Ala Phe Leu Val Gly Leu Val Cys Thr Ala Cys Val
 1 5 10 15
 Leu Leu His Asp Asp Pro Val Ala Ala Ser Thr Tyr Asn Gly Pro
 20 25 30
 Leu Ser Ser His Trp Phe Pro Glu Glu Leu Ala Gln Trp Glu Pro
 35 40 45
 35 Asp Ser Asp Pro Asp Ala Pro Phe Asn Arg Ser His Val Pro Leu
 50 55 60
 Glu Pro Gly Arg Val Ala Asn Arg Val Asn Ala Asn Ala Asp Lys
 65 70 75
 Asp Ala His Leu Val Ser Leu Ser Ala Leu Asn Arg His Thr Ser
 80 85 90
 40 Gly Val Pro Ser Gln Gly Ala Pro Val Phe Tyr Glu Asn Thr Phe
 95 100 105
 Ser Tyr Trp His Tyr Thr Asp Leu Met Val Tyr Trp Ala Gly Ser
 110 115 120
 45 Ala Gly Glu Gly Ile Ile Val Pro Pro Ser Ala Asp Val Ile Asp
 125 130 135
 Ala Ser His Arg Asn Gly Val Pro Ile Leu Gly Asn Val Phe Phe
 140 145 150
 Pro Pro Thr Val Tyr Gly Gly Gln Leu Glu Trp Leu Glu Gln Met
 155 160 165
 50 Leu Glu Gln Glu Glu Asp Gly Ser Phe Pro Leu Ala Asp Lys Leu
 170 175 180
 Leu Glu Val Ala Asp Tyr Tyr Gly Phe Asp Gly Trp Phe Ile Asn

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	185	190	195
	Gln Glu Thr Glu Gly	Ala Asp Glu Gly	Thr Ala Glu Ala Met Gln
5	200	205	210
	Ala Phe Leu Val Tyr	Leu Gln Glu Gln Lys	Pro Glu Gly Met His
	215	220	225
	Ile Met Trp Tyr Asp	Ser Met Ile Asp Thr	Gly Ala Ile Ala Trp
	230	235	240
10	Gln Asn His Leu Thr	Asp Arg Asn Lys Met	Tyr Leu Gln Asn Gly
	245	250	255
	Ser Thr Arg Val Ala	Asp Ser Met Phe Leu	Asn Phe Trp Trp Arg
	260	265	270
	Asp Gln Arg Gln Ser	Asn Glu Leu Ala Gln	Ala Leu Gly Arg Ser
	275	280	285
15	Pro Tyr Asp Leu Tyr	Ala Gly Val Asp Val	Glu Ala Arg Gly Thr
	290	295	300
	Ser Thr Pro Val Gln	Trp Glu Gly Leu Phe	Pro Glu Gly Glu Lys
	305	310	315
	Ala His Thr Ser Leu	Gly Leu Tyr Arg Pro	Asp Trp Ala Phe Gln
	320	325	330
20	Ser Ser Glu Thr Met	Glu Ala Phe Tyr Glu	Lys Glu Leu Gln Phe
	335	340	345
	Trp Val Gly Ser Thr	Gly Asn Pro Ala Glu	Thr Asp Gly Gln Ser
	350	355	360
	Asn Trp Pro Gly Met	Ala His Trp Phe Pro	Ala Lys Ser Thr Ala
	365	370	375
25	Thr Ser Val Pro Phe	Val Thr His Phe Asn	Thr Gly Ser Gly Ala
	380	385	390
	Gln Phe Ser Ala Glu	Gly Lys Thr Val Ser	Glu Gln Glu Trp Asn
	395	400	405
	Asn Arg Ser Leu Gln	Asp Val Leu Pro Thr	Trp Arg Trp Ile Gln
	410	415	420
30	His Gly Gly Asp Leu	Glu Ala Thr Phe Ser	Trp Glu Glu Ala Phe
	425	430	435
	Glu Gly Gly Ser Leu	Gln Trp His Gly Ser	Leu Ala Glu Gly
	440	445	450
35	Glu His Ala Gln Ile	Glu Leu Tyr Gln Thr	Glu Leu Pro Ile Ser
	455	460	465
	Glu Gly Thr Ser Leu	Thr Trp Thr Phe Lys	Ser Glu His Gly Asn
	470	475	480
	Asp Leu Asn Val Gly	Phe Arg Leu Asp Gly	Glu Glu Asp Phe Arg
	485	490	495
40	Tyr Val Glu Gly Glu	Gln Arg Glu Ser Ile	Asn Gly Trp Thr Gln
	500	505	510
	Trp Thr Leu Pro Leu	Asp Ala Phe Ala Gly	Gln Thr Ile Thr Gly
	515	520	525
	Leu Ala Phe Ala Ala	Glu Gly Asn Glu Thr	Gly Leu Ala Glu Phe
	530	535	540
45	Tyr Ile Gly Gln Leu	Ala Val Gly Ala Asp	Ser Glu Lys Pro Ala
	545	550	555
	Ala Pro Asn Val Asn	Val Arg Gln Tyr Asp	Pro Asp Pro Ser Gly
	560	565	570
	Ile Gln Leu Val Trp	Glu Lys Gln Ser Asn	Val His His Tyr Arg
	575	580	585
50	Val Tyr Lys Glu Thr	Lys His Gly Lys Glu	Leu Ile Gly Thr Ser
	590	595	600
	Ala Gly Asp Arg Ile	Tyr Leu Glu Gly Leu	Val Glu Glu Ser Lys

	605		610		615
5	Gln Asn Asp Val Arg	Leu His Ile Glu Ala	Leu Ser Glu Thr	Phe	
	620		625		630
	Val Pro Ser Asp Ala	Arg Met Ile Asp Ile	Lys Ser Gly Ser	Phe	
	635		640		645

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1935 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTGAGAAAAG CTTTTTTAGT CGGTCTTGT TGCACAGCGT GTGTATTGCT CCATGATGAT 60
 CCAGTTGCCG CATCTACGTA CAACGGCCCG CTGTCCTCCC ATTGGTTCC AGAGGAACCTT 120
 GCCCAATGGG AACCAAGACAG TGATCCAGAC GCACCCCTTA ACAGAAGCCA TGTTCCGCTG 180
 GAACCAGGCC GCGTTGCGAA TAGGGTAAAT GCTAATGCAG ACAAGGACGC ACACCTTGT 240
 TCGTTGTCCG CGCTAACACAG GCATACATCA GGTGTTCCAT CGCAAGGAGC GCCAGTTTC 300
 TATGAAAATA CGTTCAGCTA TTGGCATTAT ACAGATTGTA TGTTTATTG GGCTGGTTCA 360
 GCTGGCGAAG GCATTATCGT TCCGCCAAGT GCCGATGTCA TTGATGCATC GCACCGAAAT 420
 GGGGTGCCGA TTTTAGGAAA TGTGTTCTTC CCGCCGACGG TTTATGGAGG GCAGCTAGAG 480
 TGGCTAGAAC AAATGTTAGA GCAAGAGGAG GACGGTTCAT TCCCCCTTGC TGACAAATTG 540
 35 CTAGAAGTCG CAGACTATTA TGGGTTTGAC GGCTGGTTA TTAACCAAGA AACAGAAGGG 600
 GCAGACGAAG GAACAGGCCA AGCCATGCAA GCTTTCTCG TTTATTTGCA GGAACAAAAG 660
 CCAGAAGGCA TGCACATCAT GTGGTATGAC TCGATGATTG ATACAGGGGC GATGCCCTGG 720
 40 CAAAACCATT TAACGGATCG AAATAAAATG TACTTGAAA ATGGCTCGAC CCGCGTCGCT 780
 GACAGCATGT TTTTGAACCTT TTGGTGGCGT GACCAGCGCC AATCGAACGA ATTGGCACAA 840
 GCACTTGGCA GGTCTCCGTA TGACCTCTAT GCCGGAGTGG ATGTGGAAGC ACGAGGGACA 900
 45 AGTACCCCTG TTCAGTGGGA AGGCCTGTT CCTGAAGGAG AAAAGGCAGA TACATCACTC 960
 GGGTTATACC GTCCAGATTG GCCATTCAG TCAAGTGAAA CAATGGAAGC GTTTATGAA 1020
 AAAGAACTAC AATTTGGGT TGGCTCGACA GGAAATCCAG CCGAAACAGA CGGCCAGTCA 1080
 50 AATTGGCCTG GCATGGCGCA CTGGTTCCC GCGAAAAGCA CCGCTACTTC GGTACCCCTT 1140

GTGACTCACT TTAATACGGG CAGCGGCCT CAGTTTCGG CAGAAGGCAA AACTGTGTCG 1200
 GAACAGGAAT GGAATAACCG CAGCCTCAA GATGTGCTGC CGACATGGCG CTGGATTAG 1260
 5 CATGGCGGCG ATTTAGAGGC AACATTTCT TGGAAGAAG CGTTGAAGG GGGAGCTCG 1320
 TTACAATGGC ATGGCTCATT AGCGGAAGGA GAACACGCC AAATCGAGCT CTATCAAACA 1380
 10 GAGTTGCCGA TAAGCGAAGG CACTTCGCTA ACGTGGACAT TTAAAAGCGA GCACGGCAAC 1440
 GATTAAATG TGGGCTTCCG TTTAGATGGG GAAGAGGACT TCCGTTATGT GGAAGGAGAA 1500
 CAGCGTGAAT CGATAATGG TTGGACCGAG TGGACGTTGC CGCTGGATGC GTTGCTGGT 1560
 15 CAGACGATAA CAGGGCTGGC ATTTGCAGCG GAAGGGAATG AGACTGGGCT GGCAGAATTC 1620
 TATATTGGAC AACTGGCCGT AGGTGCTGAT AGCGAAAAGC CTGCCGCTCC AAACGTGAAC 1680
 GTACGCCAGT ACGACCCAGA CCCGAGTGGC ATTCAAGCTCG TATGGGAAAA ACAAAGCAAC 1740
 20 GTCCACCATT ACCCGTTA TAAAGAAACA AAGCACGGCA AAGAGCTAAT TGGCACATCT 1800
 GCTGGAGATC GAATTACCT AGAAGGCCTA GTCGAGGAAA GCAAACAAAA CGACGTGGT 1860
 CTGCATATAG AAGCACTAAG TGAAACATTT GTGCCAAGTG ATGCTCGCAT GATCGACATA 1920
 25 AAAACCGGCT CGTTT 1935

(2) INFORMATION FOR SEQ ID NO:5:

- 30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 22 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal fragment

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
 Ser Thr Tyr Asn Gly Pro Leu Ser Ser His Xaa Phe Pro Glu Glu
 1 5 10 15
 Leu Ala Gln Xaa Glu Pro Asp
 20

(2) INFORMATION FOR SEQ ID NO:6:

- 45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTTTGGATCC TTYCCNGARG ARYTNGCNCA 30

5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Ala His Leu Val Ser Leu Ser Ala Leu Asn Arg His Thr Ser
1 5 10 15
Gly Val Pro Ser Gln Gly Ala Pro Val Phe Tyr Glu Asn Thr Phe
20 25 30
Ser Tyr

20

25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTTTGAATTC ANAGTRTTYT CRTARAANAC 30

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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal fragment

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala His Thr Ser Leu Gly Leu Tyr Arg Pro Asp Trp Ala Phe Gln

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1 5 10 15
 Ser Ser Glu Thr Met Glu Ala Phe Tyr Glu Ser Leu
 20 25

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(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTTTGAATTC TCRTARAANG CYTCCATNGT YTC 33

20

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (v) FRAGMENT TYPE: internal fragment
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ser Thr Ala Thr Ser Val Pro Phe Val Thr His Phe Asn Thr Gly
 1 5 10 15
 Ser Gly Ala Gln Phe Ser Ala Glu Gly Lys
 20 25

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(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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GTTTGAATTC TGRTTRAART GNGTNACRAA 30

(2) INFORMATION FOR SEQ ID NO:13:

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 260 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGATCCTTTC CCGGAGAGCT TGCGCAATGG GAACCAGACA GTGATCCAGA CGCACCCCTTT 60
AACAGAAAGCC ATGTTCCGCT GGAACCAGGC CGCGTTGCGA ATAGGGTAAA TGCTAATGCA 120
15 GACAAGGACG CACACCTTGT TTCGTTGTCC GCGCTAAACA GGCATACATC ARGTGTTCCA 180
TCGCAAGGAG CGCCAGTTT CTATGAAAAT ACGTTCAGCT ATTGGCATTAA TACAGATTTG 240
ATGGTTTATT GGGCTGGTTC 260

(2) INFORMATION FOR SEQ ID NO:14

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 359 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

KGTGCGTGGAA CCAGCGCAA TCGAACGAAT TGCACAAGCA CTTTGGCAGG TCTCCGTATG 60
ACCTCTATGC CGGAGTGGAT GTGGAAGCAC GAGGACAAGT ACCCCKGTTTC AGTGGAAAGGC 120
35 CTGTTTCCTG AAGGAGAAAA GGCGCATACA TCACTCGGGT TATAACCGTCC AGATTGGGCA 180
TTTCAGTCAA GTGAAACAAT GGAAGCGTTT TATGAAAAAG AACTACAATT TGGGGTTGGC 240
TCGACAGGAA ATCCAGCCGA AACAGACGGC CAGTCAAATT GGCCTGGCAT GGCGCACTGG 300
40 TTTCCCGCGA AAAGCACCGC TACTTCGGTA CCCTTGTAA CTCACTTTAA CACGAATTC 359

(2) INFORMATION FOR SEQ ID NO:15

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 322 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

5 GCTATTGGCA TTATACMAGA TTTGATGGTT TATTGGGCTG GTTCAGCTGG SCGAAGNCAT 60
 TAATCGTTCC GVCCAAGTGC CGATGTCATT GATGCATCGC ACCGAAATGG GGTGCCGATT 120
 10 TTAGGAAATG TGTTCTTCCC GCCGACGGTT TATGGAGGGC AGCTAGAGTG GCTAGAACAA 180
 ATGTTAGAGC AAGAGGGAGGA CGGTTCATTC CCCCTTGCTG ACAAAATTGCT AGAAGTCGCA 240
 GACTATTATG GGTTGACGG CTGGTTTATT AACCAAGAAA CAGAAGGGC AGACGAAGGA 300
 ACAGCCGAAG CCATGCAAGC TT 322

15 (2) INFORMATION FOR SEQ ID NO:16

- (i) SEQUENCE CHARACTERISTICS:
 20 (A) LENGTH: 335 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AAGCTTTCT CGTTTATTTG CAGGAACAAA AGCCAGAAGG CATGCACATC ATGTGGTATG 60
 ACTCGATGAT TGATACAGGG GCGATCGCCT GGCAAAACCA TTTAACGGAT CGAAATAAAA 120
 30 TGTACTTGCA AAATGGCTCG ACCCGCGTCG CTGACAGCAT GTTTTGAAC TTTTGGTGGC 180
 GTGACCAGCG CCAATCGAAC GAATTGRCAC AARRCACTTG GCAGGTCTCC RTATGACCTC 240
 TADTRCCGGA GTAGATGTGG AAGCACGAGG GACAAGTACC CCTGTTCAGT GGGAAAGRCC 300
 35 GTTCCCTGAA GAGAAAGGCG CATACATVAC TCVNG 335

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Claims

1. A nucleic acid molecule encoding a polypeptide possessing endo- β -N-acetylglucosaminidase A activity selected from the group consisting of:
 - (a) nucleic acid molecules comprising the coding region of the nucleotide sequence as depicted in SEQ ID NO:2 or a fragment thereof;
 - (b) nucleic acid molecules encoding the amino acid sequence as depicted in SEQ ID NO:1, or a fragment thereof;
 - 50 (c) nucleic acid molecules encoding an amino acid sequence resulting from deletion, addition, insertion or substitution of one or more amino acids in the amino acid sequence of SEQ ID NO:1; and
 - (d) nucleic acid molecules capable of hybridizing to any one of the nucleic acid molecules of (a) to (c).
2. The nucleic acid molecule of claim 1, wherein the polypeptide is derived from bacteria of the genus Arthrobacter.
- 55 3. The nucleic acid molecule of claim 2, wherein the polypeptide is derived from Arthrobacter protoformiae strain AKU 0647 (FERM BP-4948).
4. A recombinant nucleic acid molecule which comprises a nucleic acid molecule of any one of claims 1 to 3.

5. The recombinant nucleic acid molecule of claim 4 which is a vector.
6. The vector of claim 5, wherein said nucleic acid molecule is operably linked to expression control sequences.
- 5 7. A prokaryotic or eucaryotic host cell transformed with the vector of claim 5 or 6.
8. A method for producing a polypeptide possessing endo- β -N-acetylglucosaminidase A activity, comprising the steps of:
 - 10 (a) culturing the host cell of claim 7; and
(b) recovering the polypeptide possessing endo- β -N-acetylglucosaminidase A activity from the culture obtained in step (a).
 - 15 9. A polypeptide possessing endo- β -N-acetylglucosaminidase A activity produced by the method of claim 8, or encoded by the nucleic acid molecule of any one of claims 1 to 3.
 10. An oligonucleotide or primer which specifically hybridizes with the nucleic acid molecule of any one of claims 1 to 3.
 11. An antibody or fragment thereof which specifically binds the polypeptide of claim 9.

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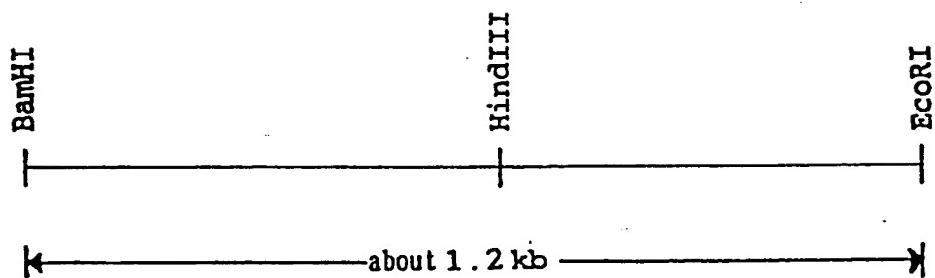


Figure 1

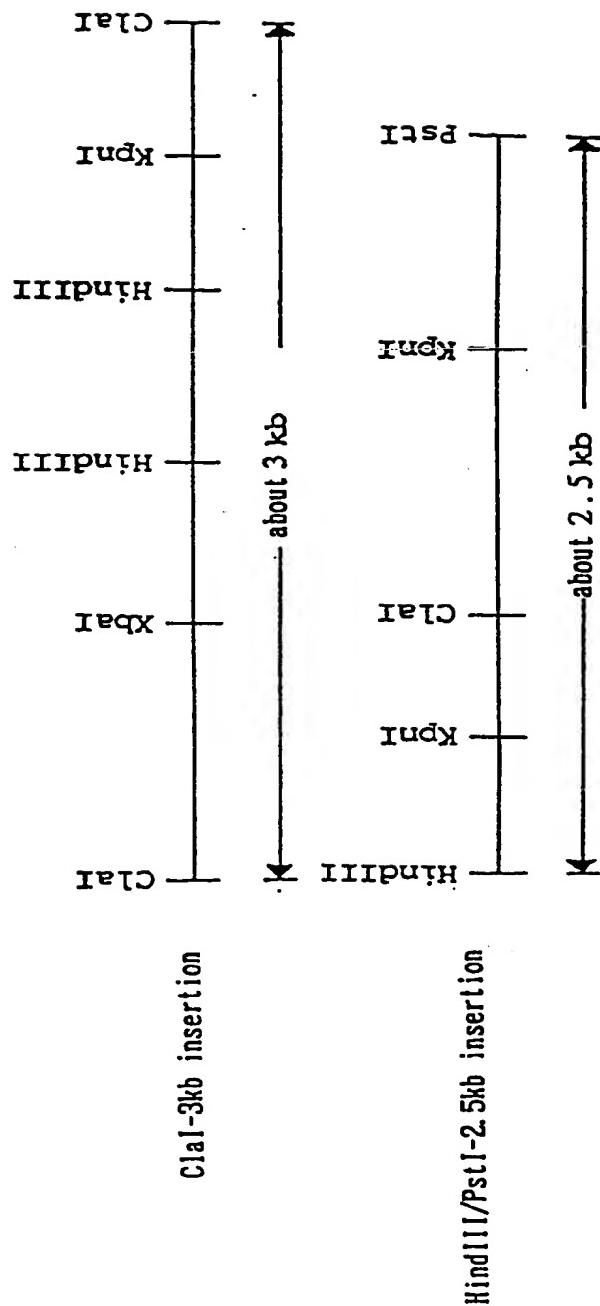


Figure 2

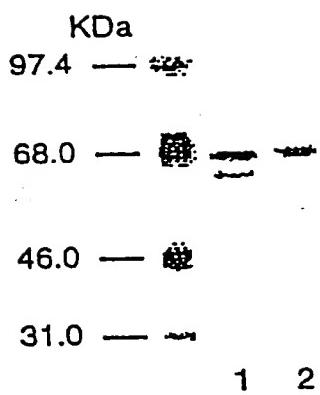


Figure 3

(19)



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(11)

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(12)

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(30) Priority: 27.10.1995 JP 303864/95

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(83) Declaration under Rule 28(4) EPC (expert
solution)

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(54) Gene encoding endo-beta-N-acetyl glucosaminidase A

(57) Described are nucleic acid molecules having a sequence encoding a polypeptide possessing endo- β -N-acetylglucosaminidase A activity or functionally equivalent variants thereof and a method for producing a polypeptide possessing endo- β -N-acetylglucosaminidase A activity or functionally equivalent variants thereof by recombinant DNA technology using the described nucleic acid molecules.



**European Patent
Office**

EUROPEAN SEARCH REPORT

Application Number

DOCUMENTS CONSIDERED TO BE RELEVANT					
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)		
X	DATABASE EMBL Accession number L36278 21. September 94 Martin, C.H. et al.: "Sequencing of the alcohol dehydrogenase (ADH) region of <i>Drosophila melanogaster</i> " XP002041599 see abstract ---	1,4,5	C12N9/78 C12N15/31 C12N15/63 C12P21/02 C12Q1/68 C07K16/40 //(C12N9/78, C12R1:06)		
X	TAKEGAWA, K. ET AL.: "Induction and purification of endo-beta-N-acetylglucosaminidase from <i>Arthrobacter protophormiae</i> grown on ovalbumin" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 55, no. 12, December 1989, pages 3107-3112, XP000654778 see page 3108, "Purification of endo-beta-GlcNAc-ase"	9			
Y	---	1-11			
Y	EP 0 539 160 A (NEW ENGLAND BIOLABS, INC.) 28 April 1993 see Example II ---	1-11	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12N		
P,X	TAKEGAWA, K. ET AL.: "Cloning, sequencing, and expression of <i>Arthrobacter protophormiae</i> endo-beta-N-acetylglucosaminidase in <i>Escherichia coli</i> " ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 338, no. 1, 1 February 1997, pages 22-28, XP002041598 see the whole document -----	1-11			
The present search report has been drawn up for all claims					
Place of search	Date of completion of the search	Examiner			
MUNICH	24 September 1997	Alt, G			
CATEGORY OF CITED DOCUMENTS					
X : particularly relevant if taken alone	T : theory or principle underlying the invention				
Y : particularly relevant if combined with another document of the same category	E : earlier patent document, but published on, or after the filing date				
A : technological background	D : document cited in the application				
O : non-written disclosure	L : document cited for other reasons				
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